

Prevalence of occult hepatitis B virus infection among the blood donors in Golestan province: cross-sectional study

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ABSTRACT

Background and Objectives: Despite the increased sensitivity of screening tests, the HBV can be transmitted during the window period and occult hepatitis B infection. The purpose of this study was to evaluate HBV markers and prevalence of OBI among HBsAg negative blood donors in Golestan province.

Materials and Methods: Anti-HBc (IgM and IgG), anti-HBs and anti-HBe tests on 4313 serum samples (HBsAg negative) were performed by ELISA method. Also, all samples for the presence of HBV- DNA were tested by using NAT methods. SPSS software and chi-square test were used for data analysis.

Results: Of the 4313 samples, 384 (8.9%) sera were anti-HBc positive. Also, of 384 anti-HBc positive samples, 302 (78.65%) were anti-HBs positive and 152 (39.6%) were anti-HBe positive. Thirty-nine (0.90%) samples were anti-HBc positive, anti-HBs negative and anti-HBe negative. HBV-DNA was not detected in any of specimens.

Conclusion: Based on the results of retesting the isolated anti-HBc samples that after one year recalling, had undetectable HBV-DNA and for the prevention of the decreasing of healthy blood donation (due to false positive anti-HBc) and preservation of the blood supplies; Individual Donor Nucleic Acid Testing (ID-NAT) along with the anti-HBc testing for the improving blood safety is recommended.

Keywords: Hepatitis B surface antigen; Blood donors; Iran; Hepatitis B infection

INTRODUCTION

Hepatitis B virus (HBV) is one of the most important causes of infection in human liver. Despite effective vaccination and antiviral treatments, HBV infection is still a public health problem worldwide. About 350 million people worldwide have chronic hepatitis B virus (1, 2). In recent years, it has made important decisions, including careful selection of

blood donors, implementation of hepatitis B vaccination and screening tests with high sensitivity and accuracy. Generally, it is believed infection with hepatitis B virus following blood transfusion remains a major concern and there is a risk of transmission of hepatitis B infection following blood transfusion (3, 4). In 1970, a new form of hepatitis B virus infection (OBI) was introduced. In OBI infection for a variety of reasons, including mutations in the S virus gene,

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HBsAg is not detectable in serum and/or plasma, and only HBV-DNA is always present in hepatocytes and sometimes in the serum of a person with a low viral load (<200 IU/ml) (5). Several factors, including host, HBV virus type, and epigenetic factors, simultaneously or separately, may be responsible for causing OBI infection, although the molecular mechanism of OBI induction is not known (6). Antibodies produced against the HBV virus core protein usually appear about 6 to 12 weeks after exposure to HBV and can be identified in the persons for a long time. The presence of anti-HBc in individual serum/plasma indicates previous infection with HBV virus in both acute and chronic phases even after virus exits the body (2, 7).

Vaccination against HBV has been available since 1982, which has been able to 95% be effective in preventing infection and progression of chronic disease and liver cancer caused by the virus. Iran is among the countries with low or medium prevalence (2.2%). However, this percentage of prevalence does not include all geographical regions of Iran and ranges from 0.87 to 8.86% in the general population (3, 8). According to published reports, there is a difference in the prevalence of HBV infection in the geographical regions of Iran. The highest prevalence of HBV in the general population is in Golestan province with 7.6-10.2% and the lowest is in Kermanshah province with 0.4-1.1% of the general population (8). The difference in prevalence of HBV infection in different provinces can be due to age, route of transmission, race, host immune status and epidemiological conditions (3, 5).

One of the factors and assumptions that may be responsible for the high prevalence of hepatitis B in Golestan province is the geographical location of the province and having a common border with Turkmenistan; they have limited contact with other parts of the country and rarely marry outside that ethnicity (8). In recent years, anti-HBc based blood donor screening has received much attention and cases of OBI in anti-HBc positive individuals have been reported (2).

Due to the importance of hepatitis B infection after blood transfusion and the high prevalence of hepatitis B infection in the population of Golestan province (8), the present study was conducted to investigate the prevalence of hepatitis B virus markers (anti-HBc, anti-HBs, anti-HBe) and OBI (HBV-DNA) among HBsAg negative blood donors in this prov-

ince, which has the highest prevalence of hepatitis B in Iranian general population.

MATERIALS AND METHODS

Study design. In 2018, 47,506 blood units were donated in Golestan province. In this cross-sectional study, 4313 samples of HBsAg negative blood donors, referred to the Golestan Blood Transfusion Center from November to February 2018, were randomly collected. Demographic data of this population were categorized and evaluated based on a questionnaire completed by donors.

Demographic data of 4313 samples of HBsAg negative were categorized and evaluated based on a questionnaire completed by donors. The study was approved by the Ethics Committee of High Institute for Research and Education in Transfusion Medicine (Number: IR.TMI.REC.1396.013). All participants were also given written consent to participate in the study before sampling. Participants were given written consent to participate in the study before sampling.

Serological tests. Initially, all 4313 blood samples that had negative results for HBsAg (Siemens, Germany), HCV Ab (Monalisa V.3, BIORAD, Germany) and HIV Ag and Ab (HIV Ag-Ab, BIORAD, USA) by ELISA method, were included in the study. Then, total anti-HBc Ab (IgM-IgG) was performed using the Enzygnost Anti-HBc Monoclonal Kit, (Siemens, Germany). All 4313 samples were tested as duplicates in order to increase the accuracy of the results and to prevent false positive results. Anti-HBs positive samples were obtained by ELISA using Enzygnost Anti-HBs II (Siemens, Germany). All anti-HBc positive samples were also tested for HBeAg & Ab by ELISA using DIA.PRO Diagnostic Bioprobes Srl kit (Milano, Italy). All ELISA tests were performed and interpreted for each kit according to the manufacturer's instructions.

HBV-DNA nested-PCR. DNA extraction on all HBsAg negative serum samples were performed using High pure viral Nucleic Acid Kit (Roche, Germany) according to the instructions in the kit. HBV Nested-PCR was performed on the extracted DNA to detect HBV genome in all samples. The Nested-PCR reaction was performed with a total volume of 25 µl, containing 12.5 µl 2× Master Mix (Takara, Japan), 0.5

μl (10 pmol/μl) of each forward and reverse primers (CinnaGen) and 5 μl (100 ng/μl) the extracted DNA. Sequences of primers used in HBV Nested-PCR assay included Primers Forward 1: 5'-CCTGCTGGTG-GCTCCAGTTC-3' and Reverse 1: 5'-CGTCCCAGC(AC) AGGATCCAGTT-3' and primers Forward 2: 5'-CYTGGCCWAAATTCGAGTCCC-3' and Reverse 2: 5'-GCAAANCCCAAAGACCACAAT-3 (9). The amplification was done using thermocycler (Corbett) with the following conditions: 1 cycle 94°C for 5 minutes and 30 cycles, 94°C for 45 sec, 63°C for 45 sec, and 72°C for 45 sec with a final extension at 72°C for 10 minutes with the ramp rate of 2.0°C/second. For the second stage of HBV Nested PCR all conditions were similar to the first stage except the binding temperature was 56°C. PCR products were visualized using 2% agarose gel electrophoresis after staining with DNA safe stain. To confirm the extraction and amplification, GAPDH gene PCR was performed on the extracted DNA of samples. The primers used in the GAPDH PCR assay include Primer Forward: 5'-ACGGATTTGGTCGTATTGGG-3' and Primer Reverse: 5'-TGATTTTGGAGG-GATCTCGC-3'. The amplification was performed with the following program: 94°C for 5 minutes for 30 cycles with denaturation temperature of 94°C 45 sec, annealing temperature of 61°C 45 sec and extension of 45 sec 72°C and final extension temperature of 10 min at 72°C (10). Also, negative and positive control plasma samples were used in each run.

Sensitivity and specificity of HBV Nested -PCR test. To determine limit of detection of HBV-DNA Nested -PCR test, hepatitis B serum sample (Opti-Quant, HBV 2E5acrometrix) with a viral particle number of 20,000 IU/ml was used, first by logarithmic method (2×10^1 IU/ml - 2×10^4) was diluted. The DNA of these diluted sera was then extracted by High pure viral Nucleic Acid Kit (Roche, Germany) and HBV Nested-PCR was performed on them. The specificity of the HBV Nested PCR assay was also confirmed by testing on 60 HBV-DNA negative sera and samples containing the genomes of HDV, HCV, CMV and HAV viruses.

Real-time PCR. For detection of HBV-DNA with another test, all anti-HBc positive samples were extracted with SpinStarViral Nucleic Acid Kit (ADT BIOTECH, Malaysia) based on instruction kit and the amplification was done using the Altona Real-time

PCR kit (Quantitative test) (Altona Diagnostics GmbH, Germany) and Rotor-Gene Q machine (QIAGEN).

Enzymatic tests. AST (Aspartate aminotransferase) and ALT (Alanine aminotransferase) enzymatic tests were performed on all HBsAg negative samples using PARS AZMUN kit, (Iran).

Isolated anti-HBc recalling. Isolated anti-HBc individuals were re-sampled after one year and serological and molecular tests were performed on the new sample as well as the AST and ALT enzymatic tests.

Statistical analysis. Analysis of data was done using IBM SPSS software package version 22.0 (IBM Corp). The Kolmogorov-Smirnov test was used to verify variables' normality of distribution. Comparisons of both groups for categorical variables were made using the χ^2 test. A P-value less than 0.05 were statistically considered as significant difference. Comparisons of both groups for categorical variables were made by the χ^2 test.

RESULTS

Of 47,506 blood donors in Golestan province 58 (0.12%) samples were HBsAg positive and 47,448 (99.88%) were HBsAg negative. In this study, 4313 samples of HBsAg negative donors were selected randomly (Table 1). The mean age of the population was 34.47 ± 10.02 and consisted of 227 (5.26%) females and 4086 (94.74%) males. According to their blood donation records, 522 (12.10%) were first-time donors, 3,791 (87.90%) were repeated and regular donors. Of the 4313 HBsAg negative samples, 384 (8.9%) were reactive as anti-HBc positive. Of 384 anti-HBc positive samples, 372 (96.87%) were male and 354 (92.1%) were regular and repeated donors. The results of anti-HBs test showed that 82 (21.35%) of samples were negative for anti-HBs and 302 (78.65%) of samples were positive for anti-HBs. Of the 302 positive anti-HBs donors, 274 (90.70%) had long-time immunity that 118 (30.75%) and 184 (47.9%) of them had antibody titers between 10-100 IU/ml and 100-600 IU/ml respectively. Results of anti-HBe test showed that 152 (39.6%) of anti-HBc positive were anti-HBe positive. Finally, out of 4313 HBsAg negative samples, 39 (0.90%) were anti-HBc positive, anti-HBs negative and anti-HBe negative that were

classified as Isolated anti-HBc. Thirty-nine Isolated anti-HBc specimens consisted of 1 (2.6%) first-time donors and 38 (97.4%) repeated and regular donors. Demographic characteristics and serologic results of anti-HBc positive donors are shown in Table 2. There was a significant relationship between the presence of anti-HBc with age (P=0.0001) (Fig. 1) and the number of anti-HBc positive samples increased with age group. Also, Spearman's correlation analysis indicated that there was a significant relationship between presence of anti-HBs and age (P = 0.009). The number of anti-HBs positive samples increased with age. In addition, there was no significant relationship between the presence of anti-HBe with age, sex and blood do-

nation status (P> 0.05). Electrophoresis of the GAPDH PCR assay showed a 230 bp band indicating the accuracy of the extraction and amplification method. The results of the nested -PCR assay on diluted sera showed LOD of 20 IU/ml of viral particles. Nested PCR product size for the positive control sample was 721 bp. The results of molecular tests (HBV Nested PCR, HBV Real-time PCR) for the presence of HBV virus genome on the samples were negative and none of these samples detected HBV-DNA. The results of AST and ALT enzymatic tests were performed on all HBsAg negative samples, according to the kit protocol used in the normal range. In HBsAg negative samples serum levels of ALT and AST enzymes were 22.64 ± 7.8U/l and 19.59 ± 6.61U/l, respectively. The results of serological and molecular tests on secondary samples of isolated anti-HBc donors showed that, similar to the results of the primary samples, they were anti-HBc positive, anti-HBs and anti-HBe negative. Serum levels of ALT and AST enzymes were in the normal range according to the Pars Aazmun KIT

Table 1. Demographic characteristics of HBsAg negative donors

Characteristics	HBsAg (-), HCV-Ab (-), HIV-Ab (-)	
	No. (%)	Total
Age, years		
20-30	1630 (37.79)	
31-40	1539 (35.69)	
41-50	883 (20.47)	
51-60	261 (6.05)	
61-65	000 (00.00)	
Gender		
Male	4086(94.73)	
Female	227(5.27)	
Marital status		
Single	793 (18.39)	
Married	3520 (81.61)	
Donor Type		
Frist time	522 (12.10)	
Repeated	884 (20.50)	
Regular	2907 (67.40)	4313 Samples

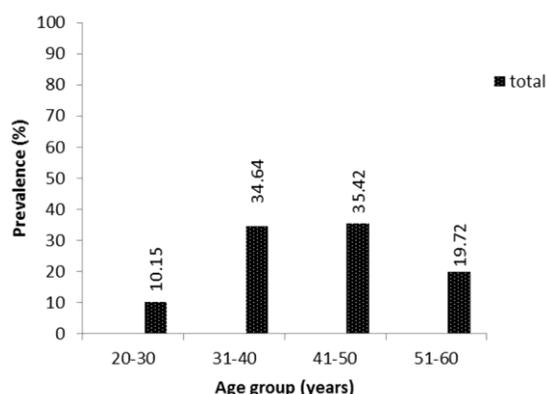


Fig 1. Distribution of anti-HBc positive seroprevalence among blood donors by age group (years).

Table 2. Demographic characteristics and serological test results of anti-HBc positive donors

Characteristics	Serological profile					
	anti-HBc (+) , 384 (100%)					
	anti-HBs (+)	anti-HBs (-)	anti-HBe (+)	anti-HBe (-)	anti-HBs (-), anti-HBe (-)	
	302 (78.65 %)	82 (21.35%)	152 (39.6%)	232 (60.4%)	39 (10.15%)	
Mean age ± SD	41.62 ± 9.29	44.57 ± 8.96	41.60 ± 8.65	42.68 ± 9.68	44.65 ± 9.08	
Gender	Female = 12	10 (3.3%)	2 (2.4%)	4 (2.6%)	8 (3.4%)	1 (2.6%)
	Male = 372	292 (96.7%)	80 (97.6%)	148 (97.4%)	224 (96.6%)	38 (97.4%)
Donor Type	First time = 30	28 (9.3%)	2 (2.45%)	10 (6.65)	20 (8.6%)	1 (2.6%)
	Repeated and	274 (90.70%)	80 (97.55%)	142 (93.55%)	212 (91.40%)	38 (87.40%)
	Regular = 354					

guidelines. There was no significant relationship between serum levels of AST, ALT, age and sex in two groups of anti-HBc positive and Isolated anti-HBc ($P > 0.05$) (Table 3). The results of molecular tests (Nested-PCR, Real-time PCR KIT) were negative for the presence of HBV virus genome among isolated anti-HBc donors, and no HBV-DNA was detected in any of these samples.

Table 3. Comparison of mean age, sex, ALT and AST levels between anti-HBc Positive and Isolated anti-HBc Donors.

	Anti-HBc Positive	Isolated Anti-HBc	P-Value
Mean age \pm SD	42.25 \pm 9.29	44.65 \pm 9.08	0.12
Gender			
Male	372 (96.9%)	38 (97.4%)	0.84
Female	12 (3.1%)	1 (2.6%)	
*ALT (U/l)	22.64 \pm 7.8	22.09 \pm 9.27	0.70
**AST (U/l)	19.59 \pm 6.61	21.43 \pm 6.50	0.19

*Alanine aminotransferase, ** Aspartate aminotransferase

DISCUSSION

Hepatitis B is one of the known factors responsible for post-transfusion infections. To reduce the risk of post-transfusion hepatitis B, all blood units donated and blood products should be screened for HBsAg (3, 11). Hepatitis B infection has been reported in the absence of HBsAg and in the presence of anti-HBc and/or anti-HBs and in some cases without the presence of any serological markers (1, 12). The prevalence of anti-HBc in different countries varies among blood donors, in the USA 0.23% (13), Italy 8.3% (7), India 10.22% (14), Turkey 21.4% (15) South Korea 13.5% (16) and Iran 16.4% among the general population and 2.1-11.5% among blood donors (17). In Iran according to different geographical regions, prevalence of anti-HBc among blood donor population varies, in our study was 8.9%, in studies, Sofian 2.1%, Behbahani 6.5%, Khamesipour 3.8% and Alizadeh 9.98% among HBsAg negative blood donors (18-21). In the USA, Japan, and several European countries, 70% of anti-HBc positive individuals have an anti-HBs titer of >100 IU/ml. Whereas in developing countries with high prevalence of HBV infection, the level of anti-HBs titre is much lower than in developed countries (2). In our study, out of 384 anti-HBc positive samples, 354 (92.1%) were regular donors and 302 (78.65%) of them had anti-HBs who 184 (47.9%)

of them had anti-HBs titers between 100-600 IU/ml. Given the high prevalence of anti-HBc in HBsAg negative blood donors in Golestan province, anti-HBc screening reduces blood donation and blood supply. To prevent the loss of blood donors in Japan, a good strategy has been designed to screen anti-HBc along with anti-HBs, and the only donors whose have anti-HBs titer >100 -500 IU/ml, eligible for blood donation (22). In 2012, Stramer released a NAT-based screening result on 6.5 million US blood donors, which showed that more than 85% of OBI infections were not detected by Multiple Donor-NAT (MD-NAT) due to the very small amount of virus and is only detectable by Individual Donor-NAT (ID-NAT) (13). Therefore, HBsAg serological testing with very high sensitivity and specificity is comparable to the MD-NAT method. Due to the problem of the financial supplies for performing ID-NAT as complementary test for reducing false negative OBI cases and disability of MD-NAT for detection of very low viral load in blood safety, so the policy of use of highly sensitive and specific HBsAg kits for screening blood donors can be effective for very high endemic area such as Golestan province. In Iran, several studies have been performed on the prevalence of HBV-DNA in HBsAg negative or anti-HBc positive blood donors. In studies of Karimi et al. in 2016, Sofian et al. in 2010, hepatitis B virus genome was not detected in HBsAg negative Iranian blood donor samples (3, 18). In our study, no HBV-DNA was detected in any of the HBsAg negative samples, indicating a concordance of our results with a number of previous studies. OBI is more commonly seen in first-time donors, but a large percentage of our study population (3791/4313) were regular donors and had the experience of blood donation as healthy donors several times. This may indicate blood safety and the lack of the presence of HBV genomes in these individuals. Recent studies have suggested that due to the very low viral load in OBI infection, despite the lack of detection of HBV-DNA in the serum, the virus genome may be present in infected hepatocytes. Accordingly, Isolated anti-HBc Blood Donors (anti-HBc Positive, anti-HBe and anti-HBs Negative), however, must be withdrawn from the blood donation cycle in spite of HBV negative DNA (23-25).

In our study 78.65% and 39.60% of anti-HBc positive individuals had anti-HBs Ab and anti-HBe Ab respectively. Only 0.90% of them were "Isolated anti-HBc". These regular donors after one year were

re-sampled then serological and molecular tests was done for these subjects. The results showed that this individuals were only anti-HBc positive and none of donors had the HBV- DNA in the sera. Therefore, to determine the immunity status of these individuals and to investigate the presence of memory cells, it is necessary that these donors receive a single dose of vaccine. The increasing anti-HBs titer could be an indicator of their safety to HBV infection. The possibility of hepatitis B transmission through blood transfusion is dependent to the volume of blood transfused, the level of HBV-DNA viral load, the presence of anti-HBs in blood donors or recipients, the recipient's immune system and the susceptibility of the recipient to infection. Therefore, vaccination of regular donors and recipients (especially immunocompromised individuals) against hepatitis B virus combined with the implementation of trace-back and look-back methods can be effective in reducing occult hepatitis B in blood transfusion and blood safety.

CONCLUSION

In our study, due to the high positivity of anti-HBc in blood donors that 0.90% of them, did not have HBV-DNA and other serologic markers of HBV after one year; so, the results of anti-HBc positivity should be truly determined. Also, due to the lack of a confirmatory test for anti-HBc test results, screening for blood donors based on this test will result in the loss of healthy blood donors and a reduction in blood reserves. Therefore, performing anti-HBc testing along with ID-NAT is necessary to increase blood safety in areas with moderate prevalence of HBV such as Iran.

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